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Responses of Potato (*Solanum tuberosum* L.) Breeding Lines to Osmotic Stress Induced in In Vitro Shoot Culture

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Abstract: In vitro experiments were conducted to study the responses of potato (*Solanum tuberosum* L.) genotypes to osmotic stress. In vitro shoot cultures of 27 breeding lines and their drought-tolerant parents (referent lines: C103 and C107) were tested under osmotic stress induced by addition of PEG 6000 (Mw = 6000; 5.0, 7.5, 10.0%, *w*/*v*), D-mannitol (0.1, 0.2, 0.3 M) and PEG 600 (Mw = 600; 2.5, 5.0, 7.5%, *w*/*v*) to the Murashige-Skoog medium. Stress index (SI) was calculated from shoot length (SL) and root length (RL), root numbers (RN) and the rate of surviving shoots (SR) ($SI_{SL;RL;RN;SR}$ = $Parameter_{SL;RL;RN;SR}$ of treated shoots/$Parameter_{SL;RL;RN;SR}$ of control shoots × 100) to compare genotypes. In the average of each breeding line and concentration, the osmotic agents resulted in SI values of 40.1, 60.8, 82.6 and 76.0 for SI_{SL}, SI_{RL}, SI_{RN} and SI_{SR}, respectively. In general, all SI values of C103 and $SI_{RL,RN}$ of C107 were significantly higher than those of the breeding lines. Nine breeding lines were found to be promising based on their final ranking. According to the results, 7.5% and 10% PEG 6000 or 0.2 M and 0.3 M D-mannitol treatments proved to be suitable for the selection of osmotic stress-tolerant genotypes.

Keywords: polyethylene glycol; D-mannitol; tissue culture; stress index; morphological traits

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1. Introduction

One of the most important abiotic stress factors is drought. Drought periods during the growing season are becoming more common due to climate change and global warming [1]. Stress caused by water deficit is even greater in areas free of permanent precipitation or where an adequate irrigation system is not available [2].

Water deficit affects almost all plant growth and developmental processes [3], can induce several morphological, physiological and biochemical changes [4], and can result in large yield losses of up to 50–70% in crop production [5].

Improving the drought tolerance and water use efficiency of varieties by using breeding methods can play an important role in reducing drought-related crop losses and thus contribute to a secure food supply for the growing population. This means that demand for drought-tolerant plant species and especially cultivars that are able to adapt to drought conditions is constantly increasing [6,7].

Potato (*Solanum tuberosum* L.) is one of the most important crops worldwide, because of its high productivity and nutritional values [8]. However, the potato crop often suffers from stress because it is sensitive to both drought [9,10] and high temperature [11]. Even though potato has difficulty tolerating water shortages due to its shallow roots [12,13], it basically manages water well.

By changes in physiology and morphology, plants are able to respond adaptively to altered environmental conditions, preceded and accompanied by cellular and molecular changes [14], so many processes are involved in the development of drought tolerance. This fact suggests that there may be large differences between species and cultivars in drought stress responses, and even variability has been found in the susceptibility of potato cultivars [15,16]. Several experiments were performed to identify traits that could be related to drought stress and to select genotypes with the desired characters in several crops [4,17] including potatoes [18–20]. Some of these traits can also be detected at the cellular and/or tissue level, such as the adjustment of osmotic pressure, which can be tested/modelled under laboratory conditions [21] or even in vitro [22,23]. Micropropagation is a commonly used process to produce virus- and disease-free plant propagating material [24], and in vitro conditions provide good opportunities to study different physiological processes and interactions and to select appropriate genotypes [25].

Osmotically active compounds such as the inert and non-penetrating polyethylene glycol (PEG) with various molecular sizes or the sugar alcohols such as D-mannitol and sorbitol are widely used to induce osmotic stress in plants [25]. Although sugars and their derivatives can significantly increase the osmotic pressure of the medium used, it must also be taken into account that they can be taken up and metabolized by plants. In addition, a high sugar content (8%) can induce tuber development in potatoes [26].

PEG is a neutral polymer, and its high molecular weight makes it suitable to imitate water deficit when added to the medium, because it cannot pass through the cell wall of the plant [27–30]. D-mannitol can be produced and metabolized by certain plant species [31]; however, it has been used efficiently in many osmotic stress tolerance experiments [25].

Numerous studies on osmotic stress tolerance of potato have been conducted in recent years. Gopal and Iwama [6] studied the effect of PEG and sorbitol on the morphological development of potato in vitro shoot cultures, and they found that in vitro tests gave results similar to those obtained under field conditions.

In addition to shoot culture, callus culture was also suitable for osmotic stress tolerance testing of genotypes, and results were well related to their field performance during drought [32]. The in vitro model provided a good basis for assessing the drought tolerance of genotypes also in other experiments [33,34]. Changes in morphological and physiological traits affected by osmotic stress are similar under in vivo conditions to those observed in in vitro tissue cultures. Survival rate, shoot length, fresh and dry weight, and number and length of roots are the most commonly affected characteristics [35–38]. Despite the fact that the growth parameters of the plantlets were strongly influenced by osmotic stress during the stress resistance experiments—in fact, they were most often inhibited—it was possible to differentiate the genotypes according to their stress tolerance only if a stress index was formed based on the measured growth parameters [32,39,40].

Although a stress index based on morpho-physiological characters alone could be used to distinguish potato genotypes [32,35,41–43], identification of quantitative trait loci (QTLs) for morphological characters [1,42] and using molecular and biochemical markers as a tool for drought tolerance study are becoming more and more widespread [1]. Our experiments were focused on the investigation of the osmotic stress tolerance of 27 potato breeding lines under in vitro conditions to reveal whether there were significant differences in SI counted from simple morphological parameters compared to the drought-tolerant referent lines. In addition, different types of osmotic agents were applied at three levels to find the effective method(s) to distinguish our breeding lines according to their osmotic stress tolerance and to select varieties that should be included in further studies. Targeted trait-specific selection started under in vitro conditions can significantly reduce the time required for development of a new variety, so the results of laboratory tests prior to the field experiment may provide a good basis for further stages of plant breeding.

2. Materials and Methods

2.1. Location of the Experiments and Plant Material

The study was conducted at the Centre for Agricultural Genomics and Biotechnology, Faculty of the Agricultural and Food Science and Environmental Management, University of Debrecen, Hungary. This study was carried out on 29 potato (*Solanum tuberosum* L.) genotypes including 27 breeding lines (C2, C3, C4, C5, C6, C8, C9, C10, C11, C12, C14, C17, C19, C20, C21, C22, C26, C28, C30, C32, C35, C37, C41, C42, C57, C58 and C63) and two drought-tolerant referent lines (C103 and C107). Selection of referent genotypes was based on their response to drought conditions in previous in vivo (field and greenhouse) experiments, in which they were proven to be drought-tolerant. C103 parental line belongs to the mid-early maturity group; its tuber is round oval, its skin is red, and the tuber flesh is yellow. C107 parental line is a late genotype with oblong, purple pink skinned tubers and white flesh. They are tolerant to late blight (*Phytophthora infestans*). All of the breeding lines tested originated from crossings of referent lines. In vitro culture establishment of breeding lines was initiated from seeds; they were surface sterilized by 3% NaOCl for 4 min, then they were washed with sterile distilled water three times. The seeds were placed onto medium containing Murashige and Skoog [44] (MS) salts and vitamins, 3% sucrose (VWR Chemicals, Radnor, PA, USA) and 0.7% agar-agar (Sigma-Aldrich, A1296, St. Louis, MO, USA) and incubated in dark at 24 °C for 5 days. Then, they were transferred to a growing room (at 22/15 ± 2 °C day/night temperature and 16 h daily illumination by 65 µmol m^{-2} s^{-1} PPF). In vitro shoot cultures of both referent lines and breeding lines were subcultured every 4 weeks on the same MS medium mentioned above.

2.2. Explant, Treatments and Culture Conditions

Single nodal cuttings, each containing an axillary bud from 4-week-old in vitro shoot cultures, were used as explant, and the shoot apex and basal part of the shoots were discarded. Explants were placed onto the basal MS medium supplemented with 3% sucrose and 0.7% agar and with polyethylene glycol (Alfa Aesar, Haverhill, MA, USA) with different molecular weights either in concentrations of 2.5, 5.0 and 7.5% or 5.0, 7.5 and 10% for PEG 600 and PEG 6000, respectively. Moreover, 0.1, 0.2 and 0.3 M D-mannitol (Merck, Burlington, VT, USA) were also applied in order to induce the osmotic stress, while the control medium was free of osmotic agent. Twenty explants per jar (450 mL, cylindrical shaped) were placed onto 50 mL of medium. Treatments consisted of five repetitions (five jars); thus, a total of 100 plantlets were observed. Experiments were repeated twice. Shoot cultures were grown under controlled conditions, in a culture room at 22/15 ± 2 °C day/night temperature and 16 h daily illumination by 65 µmol m^{-2} s^{-1} PPF for 4 weeks. At the end of experiments, the rate of survival (SR) was observed and the shoot length (SL) and the number and length of roots (RN and RL, respectively) on surviving explants were measured. Before analysis, results were expressed as percentages of the results obtained on the medium without stress agents (SI, stress index; [45]), to compare the responses to the different levels of osmotic stress in the breeding lines and referent lines.

$$SI_{SL} = \text{Shoot length of treated shoots (mm)/shoot length of control shoots (mm)} \times 100$$

$$SI_{RL} = \text{The longest root length of treated shoots (mm)/the longest root length of control shoots (mm)} \times 100$$

$$SI_{RN} = \text{Root number of treated shoots/root number of control shoots} \times 100$$

$$SI_{SR} = \text{Survival rate of treated shoots (per jar)/survival rate of control shoots (per jar)} \times 100$$

2.3. Statistical Analysis

SI calculated from morphological data (shoot and root parameters) were analyzed statistically by ANOVA followed by LSD and Tukey-B tests, using SPSS Statistics 27.0 (IBM, New York, NY, USA).

3. Results

Osmotic stress tolerance of 27 breeding lines and 2 drought-tolerant referent genotypes and their responses were compared to each other after SI values were calculated. We found that all morpho-physiological parameters were affected by treatments (Table 1), and differences were found between genotypes. Significant interactions between genotypes and type and concentration of osmotic agent were also detected.

Table 1. The main effect of osmotic agents calculated from SI values for morpho-physiological traits on the average of all potato breeding lines at different osmotic stress levels.

Traits	Level	D-Mannitol	PEG 600	PEG 6000	Mean
SL	1	68.9	55.7	45.1	
	2	39.1	32.3	39.3	
	3	23.7	18.3	38.2	
Mean		**43.9**	**35.4**	**40.9**	**40.1**
RL	1	90.5	76.4	61.5	
	2	70.0	47.5	64.9	
	3	48.3	30.1	58.0	
Mean		**69.6**	**51.3**	**61.5**	**60.8**
RN	1	93.9	80.8	96.6	
	2	88.3	62.2	99.4	
	3	75.8	41.5	105.2	
Mean		**86.0**	**61.5**	**100.4**	**82.6**
SR	1	98.5	85.9	87.8	
	2	92.5	56.1	84.8	
	3	78.5	21.7	78.4	
Mean		**89.8**	**54.6**	**83.7**	**76.0**
Total mean		**72.3**	**50.7**	**71.6**	***64.9***

Levels for D-mannitol: 1: 0.1 M, 2: 0.2 M, 3: 0.3 M; for PEG 600: 1: 2.5%, 2: 5%, 3: 7.5%; for PEG 6000: 1: 5%, 2: 7.5%, 3: 10%.

3.1. Effect of Osmotic Stress Induced by PEG 6000 on In Vitro Shoot Cultures

3.1.1. Changes in the SI of Survival Rate (SI_{SR})

SI values of survival rates (SI_{SR}) were significantly decreased or most frequently not affected by 5% PEG 6000 (Table 2, Figure S1a). Some breeding lines showed significantly lower SI_{SR} values than the referent lines. The raised level (7.5%) of PEG 6000 decreased significantly the SI_{SR} values very rarely, while other responses were similar to those observed at the level of 5% PEG (Table 2, Figure S1b). Increasing the PEG 6000 level to 10% resulted in significantly decreased SI_{SR} values for seven lines (C2, C17, C20, C28, C41, C42 and C57) compared to those observed at 7.5% (Table 2, Figure S1c). Although the C103 referent line showed the best SI_{SR} results along with two breeding lines (C8, C30), very similar results were found in eight breeding lines. However, the SI_{SR} value of the C107 referent line was significantly reduced, resulting in a number of breeding lines ahead of it.

Table 2. Survival rate SI (SI_{SR}) values of potato genotypes under osmotic stress induced by PEG 6000 added to the medium at levels of 5%, 7.5% and 10%.

Breeding Line	SI_{SR} (%)								
	5% PEG 6000			7.5% PEG 6000			10% PEG 6000		
C2	100	a A		99	a A		92	a–c B	▲
C3	87	a–d A		75	a–c A	▲□	75	a–e A	□
C4	97	ab A		75	a–c AB	▲□	60	d–f B	□
C5	93	a–c A		92	a A		83	a–e A	□
C6	63	e A	▲□	60	b–d A	▲□	74	a–e A	□
C8	100	a A		100	a A		100	a A	▲
C9	41	f A	▲□	48	d A	▲□	28	gh A	▲□
C10	95	a–c A		97	a A		92	a–c A	▲
C11	100	a A		99	a A		96	a A	▲
C12	99	a A		100	a A		96	a A	▲
C14	87	a–d A		100	a A		96	a A	▲
C17	64	e A	▲□	59	b–d A	▲□	17	h B	▲□
C19	72	de A	▲□	80	ab A	▲□	89	a–d A	
C20	100	a A		100	a A		93	ab B	▲
C21	84	a–d B	□	87	a B		98	a A	▲
C22	76	c–e B	▲□	99	a A		93	ab A	▲
C26	92	a–c A		92	a A		88	a–d A	
C28	90	a–d A		84	ab A	□	64	c–f B	□
C30	100	a A		100	a A		100	a A	▲
C32	98	ab A		96	a A		89	a–d A	
C35	89	a–d A		77	a–c A	▲□	86	a–e A	
C37	73	de A	▲□	50	d B	▲□	64	b–f B	□
C41	94	a–c A		79	ab A	▲□	44	fg B	▲□
C42	94	a–c A		96	a A		57	ef B	▲□
C57	98	ab A		84	ab A	□	62	c–f B	□
C58	79	b–e A	▲□	83	ab A	□	89	a–d A	
C63	98	ab A		54	cd B	▲□	80	a–e A	□
C103	95	a–c B		100	a A		100	a A	
C107	93	a–c A		96	a A		74	a–e A	

Symbols mark significant differences from the C107 referent line (▲), or from the C103 referent line (□) according to LSD test; the small letters indicate significantly different means between the breeding lines within a treatment, and the capital letters indicate significantly different means between treatment levels within a breeding line according to Tukey-B test.

3.1.2. Changes in the SI of Shoot Length (SI_{SL})

Each breeding line and both referent lines responded with decreased shoot length to the presence of PEG 6000 at all concentrations (Table 3, Figure S2a–c). In general, inhibition of shoot growth increased with increasing concentration of PEG 6000 to 7.5%, but higher concentrations did not result in further significant decrease. The best result (SI_{SL} 76.8) was obtained in C9 breeding line, although SI_{SL} values of some breeding lines were very similar (C2, C22, C30 and C41) (Table 3, Figure S2a). At raised PEG 6000 concentration (7.5%), five breeding lines (C2, C9, C12, C30 and C32) showed significantly higher SI_{SL} values

compared to both referent lines (Table 3, Figure S2b). At the highest level of PEG 6000 (10%), only one breeding line (C22) reached significantly higher SI_{SL} values compared to both referent lines, but most of the breeding lines performed better than the C107 referent line (Table 3, Figure S2c).

Table 3. Shoot length SI values (SI_{SL}) of potato genotypes under osmotic stress induced by PEG 6000 added to the medium at levels of 5.0%, 7.5% and 10.0%.

Breeding Line	SI_{SL} (%)								
	5% PEG 6000			7.5% PEG 6000			10% PEG 6000		
C2	65.9	a–c A	▲□	62.4	a A	▲□	56.7	ab A	▲
C3	35.2	h–l B	▲	32.3	e–g B	□	43.5	c–f A	▲□
C4	34.5	h–l A	▲	33.9	e–g A	□	32.7	g–m A	▲□
C5	24.9	lm A	□	26.9	fg A	□	30.3	j–o A	▲□
C6	32.0	i–l B		41.5	c–e A	▲	42.0	d–g A	▲□
C8	62.2	b–d A	▲□	22.8	g B	▲□	35.4	e–l C	▲□
C9	76.8	a A	▲□	64.1	a A	▲□	65.1	a A	▲□
C10	40.9	f–j A	▲	31.6	e–g B	□	28.3	k–o B	□
C11	41.4	f–i A	▲	36.5	d–f B	□	41.8	d–h A	▲□
C12	52.3	d–f A	▲□	49.2	bc A	▲□	57.8	ab A	▲
C14	34.7	h–l B	▲	40.3	c–e A	▲	34.2	f–m B	▲□
C17	28.6	j–m A	□	25.6	fg AB	□	21.9	no B	□
C19	46.1	f–h A	▲□	46.9	b–d A	▲	30.5	j–o B	▲□
C20	19.6	m C	□	31.4	e–g B	□	37.2	d–j A	▲□
C21	42.1	f–i A	▲	31.0	e–g B	□	33.0	f–m B	▲□
C22	73.7	ab A	▲□	46.3	b–d B	▲	44.8	c–e B	▲□
C26	49.5	e–g A	▲□	47.9	bc A	▲	31.1	i–n B	▲□
C28	40.5	f–j A	▲	27.3	fg B	□	25.1	l–o B	□
C30	67.8	a–c A	▲□	62.5	a A	▲□	41.5	d–i B	▲□
C32	45.0	f–h A	▲□	54.2	ab A	▲□	44.9	c–e A	▲□
C35	60.2	c–e A	▲□	32.8	e–g C	□	47.5	cd B	▲
C37	38.9	g–j A	▲	34.0	e–g B	□	31.3	h–n B	▲□
C41	68.0	a–c A	▲□	48.1	bc B	▲	40.4	d–j B	▲□
C42	46.9	f–h A	▲□	40.1	c–e B	▲	34.5	e–m B	▲□
C57	46.9	f–h A	▲□	40.9	c–e A	▲	20.7	o B	□
C58	38.1	g–k A	▲	34.8	ef A	□	37.0	d–k A	▲□
C63	52.3	d–f A	▲□	26.0	fg C	□	37.0	d–k B	▲□
C103	37.5	g–k B		42.6	c–e B		52.3	ab A	
C107	26.1	lm B		31.5	e–g A		24.4	l–o B	

Symbols mark significant differences from the C107 referent line (▲), or from the C103 referent line (□) according to LSD test; the small letters indicate significantly different means between the breeding lines within a treatment, and the capital letters indicate significantly different means between treatment levels within a breeding line according to Tukey-B test.

3.1.3. Changes in the SI of Root Length (SI_{RL})

Application of 5% PEG 6000 to medium resulted in decreased root length in each breeding line and in referent lines (Table 4, Figure S3a). SI_{RL} values of 10 breeding lines were significantly higher than those of the referent lines. At the level of 7.5% PEG 6000,

increased SI_{RL} values were found in some breeding lines, and the SI_{RL} values of four breeding lines (C10, C14, C22 and C30) were significantly higher than those of both referent lines (Table 4, Figure S3b). When 10% PEG 6000 was added to medium, the SI_{RL} value only of C22 was higher than that of referent line C103, but the SI_{RL} value of C107 was lower than that of the majority of breeding lines (Table 4, Figure S3c).

Table 4. Root length SI (SI_{RL}) values of potato genotypes under osmotic stress induced by PEG 6000 added to the medium at levels of 5.0%, 7.5% and 10%.

Breeding Line	SI_{RL} (%)								
	5% PEG 6000			7.5% PEG 6000			10% PEG 6000		
C2	50.7	f–j B		69.5	c–h A		46.8	e–i B	□
C3	56.6	d–j B		52.4	h–n B	▲□	63.6	b–e A	▲□
C4	51.1	f–j A		53.7	g–m A	▲□	51.3	d–h A	□
C5	69.4	c–f B	▲□	84.2	cd A	▲	85.1	ab A	▲
C6	48.1	h–j B		71.4	c–h A		51.8	d–h B	□
C8	83.8	a–c A	▲□	58.4	f–l B	□	72.3	b–d C	▲□
C9	87.7	ab A	▲□	62.8	e–j B		63.2	b–e B	□
C10	88.2	ab A	▲□	87.9	bc A	▲□	57.7	c–h B	□
C11	47.4	ij A	□	33.7	no B	▲□	48.0	e–i A	□
C12	47.2	ij A	□	76.7	c–f B		58.8	c–f C	▲□
C14	88.1	ab A	▲□	111.8	a B	▲□	57.0	c–g C	□
C17	44.0	f A	□	25.2	o B	▲□	17.3	k B	▲□
C19	67.5	c–h A	▲□	54.8	g–l B	▲□	39.2	f–k C	□
C20	66.6	c–h A	▲	45.0	j–o B	▲□	44.8	e–j B	□
C21	57.3	d–j A		65.4	d–i A		51.4	d–h B	□
C22	74.7	a–d B	▲□	90.0	bc A	▲□	95.9	a A	▲□
C26	49.8	g–j A	□	51.1	i–n A	▲□	24.1	jk B	▲□
C28	54.2	e–j A		41.0 B	l–o	▲□	34.5	g–k B	□
C30	91.0	a B	▲□	106.3	ab A	▲□	81.5	ab B	▲
C32	45.9	ij A	□	53.8	g–m A	▲□	36.9	f–k B	□
C35	89.0	ab A	▲□	61.5	e–k C	□	75.6	a–c B	▲
C37	55.6	e–j A		39.8	m–o B	▲□	50.3	d–h A	□
C41	52.1	f–j A		37.5	m–o B	▲□	25.7	i–k B	▲□
C42	63.6	d–i A		72.2	c–h A		32.6	h–k B	▲□
C57	45.8	d–f A		55.8	f–l A	□	20.9	k B	▲□
C58	63.2	d–j B		79.5	c–e B	▲	82.4	ab A	▲
C63	71.2	b–e A	▲□	43.0	ko B	▲□	53.7	c–h B	□
C103	56.5	d–j B		73.6	c–g B		84.7	ab A	
C107	55.4	d–j B		67.1	d–i A		46.3	e–i B	

Symbols mark significant differences from the C107 referent line (▲), or from the C103 referent line (□) according to LSD test; the small letters indicate significantly different means between the breeding lines within a treatment, and the capital letters indicate significantly different means between treatment levels within a breeding line according to Tukey-B test.

3.1.4. Changes in the SI of Root Number (SI_{RN})

PEG 6000 treatments strongly increased the number of roots in parental lines and in some of the breeding lines, and the best SI_{RN} values were obtained for referent genotypes

in each treatment (Table 5, Figure S4a–c). However, 5% PEG 6000 in the medium resulted in decreased root number in a dozen breeding lines. Some lines responded with increased SI_{RN} values to raised (7.5%) PEG concentration. Increasing the PEG 6000 concentration to 10% resulted in significantly decreased SI_{RN} values in four breeding lines (C8, C26, C41 and C57) and yielded significantly increased SI_{RN} values in about a quarter of the lines (C2, C3, C11, C30, C35, C37 and C58) (Table 5, Figure S4c).

Table 5. Root number SI (SI_{RN}) values of potato genotypes under osmotic stress induced by PEG 6000 added to the medium at levels of 5.0%, 7.5% and 10%.

Breeding Line	SI_{RN} (%)								
	5% PEG 6000			7.5% PEG 6000			10% PEG 6000		
C2	108.1	c–f B	▲□	111.9	c–e A	▲□	135.1	c A	▲□
C3	65.6	jk A	▲□	58.6	j B	▲□	82.7	e–h A	▲□
C4	101.8	c–g A	▲□	95.3	d–h A	▲□	102.6	c–g A	▲□
C5	77.6	g–k A	▲□	76.9	f–j A	▲□	80.3	e–h A	▲□
C6	60.3	k A	▲□	55.9	j A	▲□	55.9	hi A	▲□
C8	98.2	d–h C	▲□	154.9	ab A		121.2	cd B	▲□
C9	113.8	c–e A	▲□	90.1	d–i B	▲□	82.4	e–h B	▲□
C10	75.9	h–k A	▲□	80.8	f–j A	▲□	78.8	e–h A	▲□
C11	110.2	c–f A	▲□	89.7	d–i B	▲□	113.9	c–e A	▲□
C12	101.9	c–g B	▲□	117.7	cd A	▲□	110.8	c–e A	▲□
C14	90.2	e–j A	▲□	87.9	e–i A	▲□	80.6	e–h A	▲□
C17	75.3	h–k A	▲□	70.2	g–j AB	▲□	40.3	i B	▲□
C19	115.8	b–d A	▲□	101.0	d–f B	▲□	107.9	c–f A	▲□
C20	90.4	e–j A	▲□	79.3	f–j A	▲□	78.9	e–h A	▲□
C21	88.9	e–j A	▲□	97.4	d–g A	▲□	94.9	d–g A	▲□
C22	96.6	d–i A	▲□	90.0	d–i A	▲□	82.5	e–h A	▲□
C26	75.4	h–k B	▲□	86.9	e–i A	▲□	70.2	g–i B	▲□
C28	81.5	g–k A	▲□	57.8	j B	▲□	57.7	hi B	▲□
C30	70.7	i–k B	▲□	67.2	h–j B	▲□	110.2	c–e A	▲□
C32	100.8	c–h B	▲□	134.7	bc A	▲□	122.7	cd A	▲□
C35	99.5	c–h A	▲□	75.5	f–j B	▲□	111.8	c–e A	▲□
C37	120.9	b–d AB	▲□	102.1	d–f B	▲□	129.0	cd A	▲□
C41	85.6	f–j A	▲□	77.9	f–j A	▲□	51.6	hi B	▲□
C42	95.9	d–i B	▲□	111.9	c–e A	▲□	120.9	cd A	▲□
C57	124.4	bc A	▲□	129.8	c A	▲□	106.3	c–g B	▲□
C58	84.8	f–k C	▲□	96.8	d–g B	▲□	112.4	c–e A	▲□
C63	78.8	g–k A	▲□	66.4	ij B	▲□	71.3	f–i AB	▲□
C103	149.7	a B		173.0	a A		180.3	a A	
C107	138.2	ab B		161.0	ab B		220.0	b A	

Symbols mark significant differences from the C107 referent line (▲), or from the C103 referent line (□) according to LSD test; the small letters indicate significantly different means between the breeding lines within a treatment, and the capital letters indicate significantly different means between treatment levels within a breeding line according to Tukey-B test.

3.2. Effect of Osmotic Stress Induced by PEG 600 on In Vitro Shoot Cultures

3.2.1. Changes in the SI of Survival Rate (SI_{SR})

The SI_{SR} values for survival rate decreased as PEG 600 level increased (Table 6, Figure S5a–c). At the lowest (2.5%) concentration of PEG 600, the SI_{SR} values of survival rates were usually higher than 70, and some breeding lines reached 100. Only explants of C9 and C32 lines survived in significantly lower rates than referent lines. The 5% level of PEG 600 resulted in significant decreases in the survival rates of several tested breeding lines and of the referent lines. Compared to the referent lines, higher values were obtained for the C57, C58 and C19 lines, although results differed significantly only when they were compared to the C107 referent line (its SI_{SR} was 69). Moreover, C19 and C20 breeding lines showed very good survival ability, too. Similarly, significantly higher survival rates were obtained for the C20 breeding line compared to both referent lines, although the highest concentration of PEG 600 (7.5%) significantly reduced the SI_{SR} in each genotype. In contrast, most breeding lines showed lower survival rates compared to the referent lines, and most frequently the differences were significant.

Table 6. Survival rate SI (SI_{SR}) values of potato genotypes under osmotic stress induced by PEG 600 added to the medium at level of 2.5%, 5.0% and 7.5%.

Breeding Line	SI_{SR} (%)								
	2.5% PEG 600			5.0% PEG 600			7.5% PEG 600		
C2	100	a A	▲	63	a–e B	□	24	b–f C	▲□
C3	90	a–c A		55	b–e B	□	3	ef C	▲□
C4	92	ab A	▲	32	ef B	▲□	0	C	
C5	92	ab A	▲	58	b–e B	□	36	b–e B	
C6	75	a–c A	□	60	a–e A	□	15	c–f B	▲□
C8	100	a A	▲	77	a–c B		34	b–f C	□
C9	46	d A	▲□	2	f B	▲□	1	f B	▲□
C10	88	a–c A		32	ef B	▲□	5	ef C	▲□
C11	100	a A	▲	47	c–e B	▲□	27	b–f C	▲□
C12	100	a A	▲	57	b–e AB	□	34	b–f B	□
C14	89	a–c A		50	c–e B	▲□	29	b–f C	□
C17	93	ab A	▲	35	d–f B	▲□	5	ef C	▲□
C19	75	a–c A	□	88	ab A	▲	21	b–f B	▲□
C20	98	ab A	▲	87	ab AB		70	a B	▲□
C21	88	a–c A		67,5	a–d A		34	b–f B	□
C22	92	ab A	▲	58	b–e B	□	20	c–f C	▲□
C26	67	b–d A	□	34	d–f B	▲□	7	d–f C	▲□
C28	60	cd A	□	14	f B	▲□	14	c–f B	▲□
C30	100	a A	▲	64	a–e B	□	13	c–f C	▲□
C32	40	d A	▲□	6	f B	▲□	6	ef B	▲□
C35	99	a A	▲	62	a–e B	□	3	ef C	▲□
C37	94	ab A	▲	69	a–d B		30	b–f C	□
C41	74	a–c A	□	13	f B	▲□	13	c–f B	▲□
C42	100	a A	▲	76	a B		7	d–f C	▲□

Table 6. *Cont.*

Breeding Line	SI_{SR} (%)								
	2.5% PEG 600			5.0% PEG 600			7.5% PEG 600		
C57	100	a A	▲	94	a A	▲	6	ef B	▲□
C58	93	ab A	▲	95	a A	▲	40	b–d B	▲
C63	84	a–c A		78	a–c A		34	b–f B	□
C103	99	a A		87	ab B		53	bc C	
C107	74	a–c A		69	a–d A		45	b–d B	

Symbols mark significant differences from the C107 referent line (▲), or from the C103 referent line (□) according to LSD test; the small letters indicate significantly different means between the breeding lines within a treatment, and the capital letters indicate significantly different means between treatment levels within a breeding line according to Tukey-B test.

3.2.2. Changes in the SI of Shoot Length (SI_{SL})

Each breeding line and both referent lines responded with significantly decreased shoot lengths to the presence of PEG 600 at a concentration of 2.5%, and inhibition of shoot growth increased with increasing PEG 600 concentration (Table 7, Figure S6a). Referent genotypes showed very good stress tolerance compared to breeding lines. SI_{SL} values of C103 were higher than those of C107 at each level of PEG 600, and differences between SI_{SL} values of referent lines increased as PEG 600 level increased. When 2.5% PEG 600 was applied, only the SI_{SL} values of C6 and C42 were significantly higher than those of both referent lines. In addition to them, the C19 breeding line showed very good SI_{SL} results in both treatments by 2.5% and 5.0% PEG 600 (SI_{SL} were 75 and 53.9, respectively). At the highest level of PEG 600 treatment, the C103 referent line achieved the significantly best SI_{SL} result, followed by the C107 referent line, but lagging far behind.

Table 7. Shoot length SI (SI_{SL}) values of potato genotypes under stress treatment induced by PEG 600 added to the medium at levels of 2.5%, 5.0% and 7.5%.

Breeding Line	SI_{SL} (%)								
	2.5% PEG 600			5% PEG 600			7.5% PEG 600		
C2	57.9	c–g A	□	48.1	a–c B	▲	17.40	c–g C	▲□
C3	57.5	c–g A	□	36.0	b–f B	□	14.30	c–h C	▲□
C4	36.1	j A	▲□	22.3	g–l B	▲□	10.2	c–h C	▲□
C5	61.4	c–f A		28.6	e–i B	▲□	16.0	c–h C	▲□
C6	75.7	ab A	▲□	46.6	a–d B		21.3	c–f C	□
C8	68.7	a–c A		21.9	g–l B	▲□	5.0	gh C	▲□
C9	55.1	d–h A	▲□	27.5	e–j B	▲□	24.1	bc B	□
C10	41.8	h–j A	▲□	23.5	f–k B	▲□	9.7	d–h B	▲□
C11	61.4	c–f A		22.7	f–l B	▲□	14.0	c–h C	▲□
C12	24.2	k AB	▲□	29.3	e–h A	□	20.4	c–f B	▲□
C14	45.3	g–j A	▲□	28.7	e–i B	▲□	19.3	c–f C	▲□
C17	48.1	f–j A	▲□	28.1	e–i B	▲□	22.4	c–e B	□
C19	75.0	ab A	□	53.9	a B	▲	14.9	c–h C	▲□
C20	42.0	h–j A	▲□	22.5	f–l B	▲□	13.7	c–h C	▲□

Table 7. *Cont.*

Breeding Line	SI_{SL} (%)								
	2.5% PEG 600			5% PEG 600			7.5% PEG 600		
C21	63.0	b–e A	□	21.5	h–l B	▲□	17.6	c–f B	▲□
C22	51.6	e–i A	▲□	14.1	j–n B	▲□	12.0	c–h B	▲□
C26	39.0	ij A	▲□	11.6	k–m B	▲□	12.0	c–h B	▲□
C28	53.5	e–h A	▲□	15.4	i–m B	▲□	12.3	c–h B	▲□
C30	42.0	h–j A	▲□	9.5	lm B	▲□	2.8	h C	▲□
C32	18.0	k A	▲□	5.9	m B	▲□	7.4	f–h B	▲□
C35	52.3	e–i A	▲□	18.6	h–m B	▲□	10.8	c–h B	▲□
C37	51.6	e–i A	□	38.1	b–e B	□	16.0	c–h C	▲□
C41	45.6	g–j A	▲□	48.5	a–c A	▲	25.5	b A	□
C42	79.5	a A	▲□	48.4	a–c B	▲	21.7	c–e C	□
C57	52.3	e–i A	▲□	46.1	a–d A	▲	9.1	e–h B	▲□
C58	67.6	a–d A		44.1	a–d B	▲□	23.5	b–d C	□
C63	54.9	d–h A	▲□	33.6	d–h B	□	12.5	c–h C	▲□
C103	70.4	a–c A		49.3	a–c B		43.5	a B	
C107	62.7	c–f A		35.6	b–f B		25.6	b C	

Symbols mark significant differences from the C107 referent line (▲), or from the C103 referent line (□) according to LSD test; the small letters indicate significantly different means between the breeding lines within a treatment, and the capital letters indicate significantly different means between treatment levels within a breeding line according to Tukey-B test.

3.2.3. Changes in the SI of Root Length (SI_{RL})

In general, in vitro potato shoot cultures responded to PEG 600 treatment with decreased SI_{RL} values for root length, and the degree of inhibition increased with increasing PEG 600 concentration (Table 8, Figure S7a–c). The use of 2.5% PEG 600 had a stimulatory effect on the root length of four lines (C5, C20, C57 and C63) compared to the referent lines, with the highest SI_{RL} value obtained in the C63 line (131.1). Considering the SI_{RL} values for root length, the C63 breeding line was the best at each PEG 600 level. According to its response, the C5 line tolerated well the stress induced by each PEG 600 treatment, while the C57 and C20 lines tolerated only the mild stress (2.5% PEG 600) according to their SI_{RL} values for root length. However, the majority of breeding lines showed lower SI_{RL} results than those of the referent lines for root length in each PEG treatment, especially when PEG 600 was applied in the highest (7.5%) concentration.

Table 8. Root length SI (SI_{RL}) values of potato genotypes under osmotic stress induced by PEG 600 added to the medium at levels of 2.5%, 5.0%, and 7.5%.

Breeding Line	SI_{RL} (%)								
	2.5% PEG 600			5% PEG 600			7.5% PEG 600		
C2	57.7	h–j A	▲□	31.1	e–j B	▲□	8.5	de C	▲□
C3	54.0	h–k A	▲□	35.2	d–i B	▲□	13.9	c–e B	▲□
C4	64.7	g–i A	▲□	27.3	e–k B	▲□	0		
C5	99.5	bc A	▲	92.3	a A	▲	46.7	a–d B	□
C6	89.9	c–e A		47.9	b–g B	▲□	21.0	c–e C	▲□

Table 8. *Cont.*

Breeding Line	SI_{RL} (%)								
	2.5% PEG 600			5% PEG 600			7.5% PEG 600		
C8	73.9	f–h A	▲□	26.0	f–k B	▲□	3.7	de C	▲□
C9	50.3	i–l A	▲□	23.1	h–k B	▲□	0		
C10	75.9	fg A	□	37.9	c–h B	▲□	10.6	de B	▲□
C11	93.4	b–d A		25.5	g–k B	▲□	14.5	c–e C	▲□
C12	51.8	h–l AB	▲□	66.1	b A	□	27.4	b–e B	▲□
C14	92.4	b–e A		25.4	g–k B		9.3	de B	▲□
C17	61.7	g–j A	▲□	26.2	f–k B	▲□	21.4	c–e B	▲□
C19	79.0	ef A	□	56.4	b–d B	□	9.7	de C	▲□
C20	105.6	b A	▲	47.3	b–g B	▲□	18.6	c–e C	▲□
C21	81.3	d–f A	□	26.9	f–k B	▲□	21.71	c–e B	▲□
C22	57.5	h–j A	▲□	17.5	h–k B	▲□	15.4	c–e B	▲□
C26	39.2	l A	▲□	4.8	k B	▲□	1.6	e B	▲□
C28	57.9	h–j A	▲□	13.5	i–k B	▲□	6.1	de B	▲□
C30	49.0	i–l A	▲□	8.9	jk B	▲□	2.5	e B	▲□
C32	23.6	m A	▲□	5.2	k B	▲□	2.8	e B	▲□
C35	74.8	f–h A	□	31.1	e–j B	▲□	7.1	de B	▲□
C37	64.9	g–i A	▲□	50.0	b–f B	□	11.5	de C	▲□
C41	41.1	kl A	▲□	38.0	c–h A	▲□	15.8	c–e B	▲□
C42	74.5	f–h A	□	37.8	c–h B	▲□	16.2	c–e C	▲□
C57	105.6	b A	▲□	50.9	b–e B	□	11.7	de C	▲□
C58	81.7	d–f A	□	59.0	bc B	□	44.1	a–e C	□
C63	131.1	a A	▲□	107.8	a B	▲□	73.4	a C	▲
C103	98.6	bc A		87.1	A		64.2	ab B	
C107	84.9	de A		59.7	B		54.2	a–c B	

Symbols mark significant differences from the C107 referent line (▲), or from the C103 referent line (□) according to LSD test; the small letters indicate significantly different means between the breeding lines within a treatment, and the capital letters indicate significantly different means between treatment levels within a breeding line according to Tukey-B test.

3.2.4. Changes in the SI of Root Number (SI_{RN})

The number of roots decreased in the majority of breeding lines when media contained PEG 600, and the SI values decreased as PEG 600 level increased (Table 9, Figure S8a–c). However, SI values of higher than 100 were obtained for the C2 and C3 breeding lines in the 2.5% PEG 600 treatment, and their results were significantly higher compared to those of the referent lines and the majority of other breeding lines when treated with 2.5 and 5.0% PEG 600. In addition, the C42 breeding line also achieved significantly higher SI results with the 5.0% PEG treatment. When 7.5% PEG 600 was added to the medium, the C103 referent line showed the significantly best result (72.2), while the SI value of the other referent line (C107) was significantly lower (57.7) than that of C103, but significantly higher than those of most breeding lines. Moreover, the C2 and C3 breeding lines showed results very similar to those of the C107 referent line.

Table 9. Root number SI (SI_{RN}) values of potato genotypes under osmotic stress induced by PEG 600 added to the medium at levels of 2.5%, 5.0%, and 7.5%.

Breeding Line	SI_{SN} (%)								
	2.5% PEG 600			5% PEG 600			7.5% PEG 600		
C2	109.3	a A	▲□	103.0	a A	▲□	58.9	ab B	□
C3	108.0	a A	▲□	90.7	a–c B	▲□	62.2	ab B	
C4	82.6	b–e A	□	57.9	c–f B	▲□	0.0		
C5	74.2	c–g A	□	72.2	a–e A	□	56.7	ab B	□
C6	59.2	gh A	▲□	45.1	d–f B	▲□	24.2	ab C	▲□
C8	70.7	d–h A	▲□	47.4	d–f B	▲□	22.5	ab C	▲□
C9	84.8	b–d		0.0			0.0		
C10	74.6	c–h A	□	53.7	d–f B	□	22.7	ab C	▲□
C11	96.7	ab A	▲	48.9	d–f B	▲□	35.3	ab B	▲□
C12	50.7	h A	▲□	43.2	d–f A	▲□	28.4	ab B	▲□
C14	81.5	b–e A	□	59.8	c–f B	□	35.7	ab C	▲□
C17	93.9	a–c A		62.7	b–f B		0.0		
C19	76.2	c–g A	□	52.7	d–f B	□	27.7	ab C	▲□
C20	51.8	h A	▲□	41.9	d–f B	▲□	34.4	ab C	▲□
C21	70.9	d–h A	▲□	52.9	d–f B	□	38.4	ab C	▲□
C22	71.5	d–h A	▲□	47.8	d–f B	▲□	24.2	ab C	▲□
C26	78.3	c–g A	□	33.5	f B	▲□	29.9	ab B	▲□
C28	77.2	c–g A	□	37.2	ef B	▲□	34.5	ab B	▲□
C30	91.9	a–d A		32.4	f B	▲□	25.4	ab B	▲□
C32	62.1	f–h A	▲□	33.6	f A	▲□	38.4	ab A	□
C35	77.7	c–g A	□	41.7	d–f B	▲□	13.6	b B	▲□
C37	91.8	a–d A		52.5	d–f B	□	28.8	ab C	▲□
C41	71.6	d–h A	□	52.8	d–f A	□	29.5	ab B	▲□
C42	96.7	ab A	▲	95.9	ab A	▲□	31.3	ab B	▲□
C57	65.5	e–h B	▲□	75.5	a–d A	▲	47.3	ab B	▲□
C58	80.2	b–f A	□	73.3 B	a–e	▲	41.6	ab C	▲□
C63	84.8	b–d A		58.6	c–f B	□	28.8	ab C	▲□
C103	94.3	a–c A		73.0	a–d B		72.2	ab B	
C107	83.6	b–d A		61.4	c–f B		57.7	ab B	

Symbols mark significant differences from the C107 referent line (▲), or from the C103 referent line (□) according to LSD test; the small letters indicate significantly different means between the breeding lines within a treatment, and the capital letters indicate significantly different means between treatment levels within a breeding line according to Tukey-B test.

3.3. Effect of Osmotic Stress Induced by D-Mannitol in In Vitro Shoot Cultures

3.3.1. Changes in the SI of Survival Rate (SI_{SR})

The survival rates of the shoot cultures were not significantly affected by any concentration of D-mannitol in the case of about 41% of the breeding lines, and in fact, each explant survived in all treatments in five breeding lines (C2, C8, C20, C30 and C63) (Table 10, Figure S9a–c). Using D-mannitol at a concentration of 0.1 M resulted in significantly lower survival in C9, C21, and C58 and in referent line C107 than in referent line C103. A remarkable number of the breeding lines showed significantly higher SI_{SR} values than the C107

referent line. In the treatment with 0.2 M D-mannitol, significantly lower SI_{SR} values were observed in three breeding lines (C6, C22 and C35) and the C107 referent line compared to their respective values obtained with the 0.1 M and 0.2 M D-mannitol levels. When 0.3 M D-mannitol was applied, decreased SI_{SR} values were found in 12 breeding lines and in the C103 referent line. Moreover, all tested genotypes showed significantly higher SI_{SR} values compared to the C107 referent line.

Table 10. Survival rate SI (SI_{SR}) values of potato genotypes under stress treatment induced by D-mannitol added to the medium at levels of 0.1 M, 0.2 M and 0.3 M.

Breeding Line	SI_{SR} (%)								
	0.1 M D-Mannitol			0.2 M D-Mannitol			0.3M D-Mannitol		
C2	100.0	a A	▲	100.0	a A	▲	100.0	a A	▲□
C3	100.0	a A	▲	100.0	a A	▲	74.0	a–f B	▲
C4	96.0	a A		91.0	ab A		54.0	e–g B	
C5	100.0	a A	▲	95.0	ab AB		89.0	a–d B	▲□
C6	100.0	a A		67.0	c B	▲□	54.0	e–g B	
C8	100.0	a A	▲	100.0	a A	▲	100.0	a A	▲□
C9	90.0	a A	□	83.0	a–c A	□	73.0	a–f A	▲
C10	100.0	a A	▲	100.0	a A	▲	92.0	a–d B	▲□
C11	100.0	a A	▲	95.0	ab A		92.0	a–d A	▲□
C12	100.0	a A	▲	97.0	a A		74.0	a–f B	▲
C14	100.0	a A	▲	94.0	ab A		77.0	a–e B	▲
C17	100.0	a A	▲	90.0	a–c AB		75.0	a–f B	▲
C19	96.0	a A		94.0	ab A		89.0	a–d A	▲□
C20	100.0	a A	▲	100.0	a A	▲	100.0	a A	▲□
C21	91.3	a A	□	83.0	a–c A	□	51.0	e–g B	□
C22	96.0	a A		83.0	a–c B	□	56.0	e–g C	
C26	100.0	a A	▲	96.0	a A		76.0	a–f B	▲
C28	100.0	a A	▲	100.0	a A	▲	98.0	ab A	▲□
C30	100.0	a A	▲	100.0	a A	▲	100.0	a A	▲□
C32	99.0	a A	▲	100.0	a A	▲	92.0	a–d A	▲□
C35	100.0	a A	▲	88.0	a–c B		72.0	b–f C	▲
C37	100.0	a A	▲	94.0	ab A		96.0	a–c A	▲□
C41	100.0	a A	▲	85.0	a–c A	□	50.0	fg B	□
C42	100.0	a A	▲	92.0	ab A		68.0	d–g B	▲
C57	100.0	a A	▲	96.0	a A		93.0	a–d A	▲□
C58	87.0	a A	□	73.0	bc A	□	70.0	c–f A	▲
C63	100.0	a A	▲	100.0	a A	▲	100.0	a A	▲□
C103	100.0	a A		100.0	a A		68.0	d–g B	
C107	90.0	a A		85.0	a–c A		44.0	g B	

Symbols mark significant differences from the C107 referent line (▲), or from the C103 referent line (□) according to LSD test; the small letters indicate significantly different means between the breeding lines within a treatment, and the capital letters indicate significantly different means between treatment levels within a breeding line according to Tukey-B test.

3.3.2. Changes in the SI of Shoot Length (SI_{SL})

The presence of D-mannitol at a concentration of 0.1 M in medium inhibited the shoot growth of almost all genotypes, and in general, SI_{SL} values decreased significantly with increasing D-mannitol concentration (Table 11, Figure S10a–c). The SI_{SL} value of the C12 line was higher than 100 in the treatment with 0.1 M D-mannitol, and 13 breeding lines achieved significantly higher SI_{SL} values than both referent lines. Similarly, at 0.2 M and 0.3 M levels of D-mannitol, the SI_{SL} values of several breeding lines were significantly higher than those of the referent lines.

Table 11. Shoot length SI (SI_{SL}) values of potato genotypes under stress treatment induced by D-mannitol added to the medium at levels of 0.1 M, 0.2 M and 0.3 M.

Breeding Line	SI_{SL} (%)								
	0.1 M D-Mannitol			0.2 M D-Mannitol			0.3 M D-Mannitol		
C2	54.4	h–l A		34.6	g–j B	□	23.0	d–g C	▲
C3	67.4	e–g A	▲□	37.2	f–i B		21.1	e–i C	▲
C4	65.4	e–i A	▲	32.4	h–k B	□	11.7	k C	□
C5	65.4	g–l A		29.2	i–k B	▲□	24.5	c–f B	▲
C6	53.0	i–l A	□	33.6	h–j B	□	21.4	e–h C	▲
C8	93.2	b A	▲□	46.8	a–e B	▲□	24.2	c–f C	▲
C9	60.4	f–l A	▲	46.9	a–e B	▲□	29.5	bc C	▲□
C10	52.4	j–l A	□	27.3	jk B	▲□	20.8	e–i C	▲
C11	68.3	e–g A	▲□	36.1	g–i B	□	19.0	f–i C	▲□
C12	119.7	a A	▲□	51.8	ab B	▲□	36.1	a C	▲□
C14	89.0	bc A	▲□	52.6	ab B	▲□	40.9	a C	▲□
C17	74.1	de A	▲□	54.7	a B	▲□	38.0	a C	▲□
C19	87.4	bc A	▲□	39.4	e–i B		27.5	b–d C	▲□
C20	56.0	g–l A		24.4	k B	▲□	15.3	i–k C	□
C21	57.9	g–l A		28.5	i–k B	▲□	16.3	h–k C	□
C22	71.4	d–f A	▲□	37.0	g–i B		21.5	e–h C	▲
C26	70.8	d–f A	▲□	49.8	a–c B	▲□	25.5	b–e C	▲
C28	66.5	e–h A	▲	41.1	d–h B	▲	28.1	b–d C	▲□
C30	67.8	e–g A	▲□	35.0	g–j B	□	17.7	g–j C	▲□
C32	75.1	de A	▲□	48.1	a–d B	▲□	30.0	bc C	▲□
C35	59.2	f–l A	▲	32.5	h–k B	□	15.6	h–k C	□
C37	53.2	i–l A	□	34.3	g–j B	□	31.0	b B	▲□
C41	65.0	e–j A	▲	45.6	b–f B	▲	26.2	b–f C	▲
C42	51.7	kl A	□	30.2	i–k B	□	17.9	i–k C	▲□
C57	47.5	l A	□	37.2	f–i B		21.3	f–i C	
C58	81.2	cd A	▲□	46.5	a–e B	▲□	24.7	a–e C	▲
C63	89.3	bc A	▲□	42.5	c–g B	▲	25.1	c–g C	
C103	60.6	g–k A		41.1	d–h B		23.3	d–g C	
C107	51.0	kl A		34.9	g–j B		13.2	jk C	

Symbols mark significant difference from the C107 referent line (▲), or from the C103 referent lines (□) according to LSD test; the small letters indicate significantly different means between the breeding lines within a treatment, and the capital letters indicate significantly different means between treatment levels within a breeding line according to Tukey-B test.

3.3.3. Changes in the SI of Root Length (SI_{RL})

About half of the breeding lines responded to 0.1 M D-mannitol with significant decreases in their root length, but the SI_{RL} values of two breeding lines were significantly increased (Table 12, Figure S11a). One of them (C63) showed significantly a higher result than the C103 referent line, although the C103 referent line also responded with increased root length. However, as D-mannitol concentration increased, the SI_{RL} values decreased, mostly significantly (Table 11, Figure S10a–c). When 0.2 M D-mannitol was applied, only the C103 referent line showed SI_{RL} values higher than 100, but the SI_{RL} values of C17 and C63 breeding lines did not differ significantly. Decreased SI_{RL} values were found at this level of D-mannitol in almost all breeding lines. SI_{RL} values of breeding lines C17 and C63 were significantly higher than those of the C107 referent line, and increased SI_{RL} values were detected in more than half the of breeding lines. At the highest D-mannitol level (0.3 M), each genotype involved in the experiment showed decreased SI_{RL} values, and we found significantly higher SI_{RL} values in more than a third of the breeding lines compared to the C103 referent line.

Table 12. Root length SI (SI_{RL}) values of potato genotypes under stress treatment induced by D-mannitol added to the medium at levels of 0.1 M, 0.2 M and 0.3 M.

Breeding Line	SI_{RL} (%)								
	0.1 M D-Mannitol			0.2 M D-Mannitol			0.3 M D-Mannitol		
C2	97.9	b–f A	▲□	61.0	e–h B	▲□	37.6	j–m C	▲
C3	86.8	e–i A	□	59.0	e–h B	▲□	37.6	j–m C	▲
C4	74.0	i–k A	▲□	48.8	gh B	▲□	18.4	o C	▲□
C5	86.5	e–i A	□	73.3	c–e B	□	78.5	a B	□
C6	59.2	k A	▲□	50.5	gh B	▲□	34.5	k–n C	▲□
C8	101.9	b–e A	▲	84.6	bc B	□	70.0	a–c C	□
C9	81.0	f–j A	□	77.8	cd A	□	39.6	i B	▲
C10	105.1	a–d A	▲	70.0	c–e B	□	48.5	f–k C	▲
C11	90.0	d–i A	□	79.3	cd B	□	60.3	b–f C	□
C12	94.5	c–g A	▲□	69.3	c–e B	▲□	56.5	c–h C	▲□
C14	93.7	c–g A	□	50.6	gh B	▲□	21.7	no C	▲□
C17	95.2	c–g A	▲□	97.7	ab A	▲	72.6	ab B	□
C19	92.4	c–g A	□	60.3	e–h B	▲□	44.3	f–l C	▲
C20	99.1	b–e A	▲□	74.3	c–e B	□	55.9	c–i C	▲□
C21	67.2	jk A	▲□	48.6	gh B	▲□	30.3	l–o C	▲□
C22	67.8	jk A	▲□	46.0	h B	▲□	37.8	j–m B	▲
C26	91.4	d–h A	□	67.2	d–f B	▲□	27.5	m–o C	▲□
C28	84.7	e–i A	□	52.5	f–h B	▲□	43.7	f–h C	▲
C30	74.7	h–k A	▲□	68.9	c–e A	▲□	39.8	i–m B	▲
C32	91.3	d–h A	□	70.2	c–e B	□	57.9	b–g C	□
C35	78.1	g–j A	□	49.4	gh B	▲□	37.2	j–m C	▲
C37	96.6	b–f A	▲□	68.6	c–e B	▲□	47.1	f–k C	▲
C41	112.7	ab A	▲	63.9	d–h B	▲□	41.4	h–m C	▲
C42	86.2	e–i A	□	69.1	c–e B	□	48.3	f–k C	▲

Table 12. *Cont.*

Breeding Line	SI_{RL} (%)								
	0.1 M D-Mannitol			0.2 M D-Mannitol			0.3 M D-Mannitol		
C57	104.8	a–d A	▲	84.2	bc B	□	64.3	a–e C	□
C58	88.6	d–i A	□	78.9	cd B	□	52.0	e–j C	▲□
C63	119.9	a A	▲□	96.5	ab B	▲	53.0	d–j C	▲□
C103	108.8	ab A		103.7	ab A		44.2	g–l B	
C107	85.4	e–i A		77.9	cd A		68.4	a–c A	

Symbols mark significant differences from the C107 referent line (▲), or from the C103 referent line (□) according to LSD test; the small letters indicate significantly different means between the breeding lines within a treatment, and the capital letters indicate significantly different means between treatment levels within a breeding line according to Tukey-B test.

3.3.4. Changes in the SI of Root Number (SI_{RN})

About half of the breeding lines responded with decreased SI_{RN} values to 0.1 M D-mannitol treatment (Table 13, Figure S12a). Some breeding lines showed significantly increased SI_{RN} values compared to both referent lines. In general, decreasing tendencies could be observed in SI_{RN} values as D-mannitol level increased (0.2 M), and significant reductions were verified in the majority of breeding lines. SI_{RN} values increased in some breeding lines. There were significant differences between C103 and C107 when 0.1 M and 0.2 M D-mannitol concentrations were applied to the medium. In addition, four, eleven and nine breeding lines showed significantly higher SI_{RN} values compared to both referent lines at the levels of 0.1, 0.2 and 0.3 M D-mannitol, respectively (Table 13, Figure S12a–c).

Table 13. Root number SI (SI_{RN}) values of potato genotypes under stress treatment induced by D-mannitol added to the medium at levels of 0.1 M, 0.2 M and 0.3 M.

Breeding Line	SI_{RN} (%)								
	0.1 M D-Mannitol			0.2 M D-Mannitol			0.3 M D-Mannitol		
C2	91.4	d–j A	□	74.4	g–j B		67.6	f–i B	
C3	91.2	d–k A	□	75.6	f–j B		43.2	kl C	▲□
C4	88.3	e–k A	□	75.7	f–j B		50.7	i–l C	▲□
C5	113.2	b A	▲□	93.1	c–h B	▲	97.5	bc B	▲□
C6	79.6	i–l A	▲□	65.4	ij B	▲□	77.7	c–g AB	
C8	108.0	b–d A	▲	100.9	cd A	▲□	77.0	d–h B	
C9	87.2	e–k A	□	85.5	d–i A		64.4	g–j B	
C10	97.2	b–i A		79.4	e–i B		63.0	g–k C	
C11	80.5	h–l B	▲□	121.2	b A	▲□	121.7	a A	▲□
C12	80.5	h–l A	▲□	67.7	ij B	□	45.0	j–l C	▲□
C14	108.5	b–d A	▲	94.7	c–f B	▲□	70.6	f–i C	
C17	111.1	bc A	▲□	104.9	bc A	▲□	98.4	b A	▲□
C19	114.6	b A	▲□	82.8	d–i B		83.8	b–g B	▲□
C20	68.0	l A	▲□	73.3	h–j A		55.7	h–j B	□
C21	72.9	kl A	▲□	58.7	j B	▲□	55.9	h–k B	□
C22	99.2	b–h A		94.6	c–g A	▲□	49.9	i–l B	▲□

Table 13. *Cont.*

Breeding Line	SI_{RN} (%)								
	0.1 M D-Mannitol			0.2 M D-Mannitol			0.3 M D-Mannitol		
C26	98.5	b–h A		95.3	c–f A	▲□	69.6	f–i B	
C28	94.3	c–j A		93.2	c–h A	▲□	74.1	eh B	
C30	105.3	b–e B	▲	119.4	b A	▲□	96.5	b–d B	▲□
C32	134.0	a B	▲□	173.2	a A	▲□	135.2	a B	▲□
C35	82.0	g–l A	□	96.8	c–e A	▲□	64.6	g–j B	
C37	88.1	e–k A	□	75.4	f–j B		66.7	f–i B	
C41	76.3	j–l A	▲□	59.0	j B	▲□	35.2	l C	▲□
C42	78.8	i–l A	▲□	73.7	h–j A		56.3	h–k B	□
C57	82.3	g–l A	□	85.6	c–i A		85.9	b–f A	▲□
C58	105.1	b–e A	▲	104.8	bc A	▲□	87.7	b–f B	▲□
C63	84.2	f–l B	□	83.3	d–i B		91.4	b–e A	▲□
C103	100.9	b–g A		82.4	d–i B		68.8	f–i C	
C107	90.5	d–j A		76.6	f–i B		67.0	f–i B	

Symbols mark significant differences from the C107 referent line (▲), or from the C103 referent line (□) according to LSD test; the small letters indicate significantly different means between the breeding lines within a treatment, and the capital letters indicate significantly different means between treatment levels within a breeding line according to Tukey-B test.

4. Discussion

In conventional breeding work including field experiments, the test for stress tolerance of breeding lines is expensive, takes a long time [46], and the results obtained in different experiments can be variable because the reaction of the plants is strongly influenced by environmental conditions [47]. That is why several new approaches are applied to evaluate traits that can be associated with drought tolerance, including laboratory mo-dels and biotechnological tools. Responses of several potato varieties to osmotic stress under in vitro conditions matched well with the drought tolerance results obtained in the field experiments [33,34]. In addition, if we take into account the very large number of offspring produced by crosses to be screened for the desired traits, the importance of alternative experiments cannot be ignored.

In field and greenhouse and also pot experiments, drought affected all physiological and agronomic traits of the potato cultivars studied [35,36]. Osmotic stress applied to model drought stress under in vitro conditions also resulted in significant changes in all studied traits in potato, e.g., survival rate, shoot length, fresh and dry weight, and root number and length [37,38]. However, no difference could be revealed between genotypes if the evaluation was based only on the measured parameters, even though significant inhibition of the growth of in vitro shoots and roots as an effect of osmotic stress in *Solanum* species was observed [32,39]. The development of a multi-parameter stress index (SI), when the results were expressed as a percentage of the values for the controls, allowed a differentiation of genotypes according to their stress tolerance [32,40]. Accordingly, a stress index was formed from our results for each treatment examined, and the examined genotypes were evaluated based on their SI values.

In our experiments, the responses of potato breeding lines were compared both to each other and to two referent lines. We observed that the osmotic stress resulted in explant deaths in varying proportions in the breeding lines and the referent lines, most often without shoot development. High survival rates were found in C2, C8, C20, C30, and C63 in the treatments with D-mannitol, in C8 and C30 in the treatments with PEG 6000, and in C8, C12, C20, C57, C58 and C63 in the treatments with PEG 600. After ranking the breeding lines based on their SI values, nine genotypes (in descending order: C8, C63, C14,

C2, C5, C58, C12, C11 and C30) were found to be valuable breeding material (Tables S1–S4). However, the C103 referent line was the 1st in the ranking, while C107 was the 11th.

Considering the responses of referent genotypes to osmotic stress, they showed (sometimes significantly) different SI results. In general, SI results of C103 were higher than those of C107 in almost all treatments and for almost all traits studied.

It could be supposed from these results that drought tolerance of referent lines could be based on a different mechanism. Although osmotic adjustment ability in potato was found to be restricted to 0.16 MPa [48], its role in drought tolerance was reported for several crops [49]. Osmotic stress tolerance expressed at the tissue level may play a greater role in referent genotype C103, while other factors should be considered in the case of the C107 genotype. As drought tolerance is rather complex in nature, other factors, for example, regulation of stoma closure [16,50] and/or phenological properties (especially at time of maturity) could be relevant [14,51,52]. However, considering our results, morphological factors including root developmental characters formed under stress conditions can be of great importance [53]. All of these traits can help prevent dehydration of tissues either via reduction of water loss or increase in water uptake [14,52].

Simple morpho-physiological traits were proven to be suitable parameters for distinguishing genotypes according to their osmotic stress tolerance [32,35,41–43]. Although several biochemical markers [46,53,54] and QTLs [42] were found to be exact tools for the selection of drought-tolerant genotypes, when laboratory infrastructure and/or budget are limited, researchers are forced to use simple parameters. Therefore, we observed shoot and root length, number of roots, and survival rate and found that morpho-physiological responses of potato shoot cultures to osmotic stress included both the adaptive and—most frequently—the damage responses. Phenomena of reduced growth of shoot and root were mild physiological damage responses, while strong osmotic stress often resulted in explant death. However, several breeding lines showed increased root length and/or root number, maybe as an adaptive response that could play an important role in drought to-lerance and lead to plant escape from water stress [14].

The underground part of the plant (the root system), in addition to providing a site for fixation and water and nutrient uptake, plays a significant role in evolving abiotic stress responses [17,53]. Prolonged drought can induce adaptive responses in plants, and it can be manifested by modified structure and function of roots [53], which can play an important role in coping with water stress [19,35,55].

Although the morphology, function and even histology of roots can be highly varied in plants developed in the field, in growing containers, in hydroculture systems or in vitro [53,56], the separation of genotypes based on their rooting characters in laboratory tests can be applied in breeding work [57]. The relationship between root growth and water uptake was demonstrated by Iwama [58] under both in vitro and field conditions.

In our experiments many breeding lines responded to osmotic stress with longer and/or more root development, while others showed varying degrees of inhibited growth. In general, the lower concentrations of osmotic agent resulted in longer roots and, most frequently, more roots developed on shoots compared to the control culture. The root length SI results for the C103 referent line were very high under several osmotic treatments. Both referent lines developed the most roots under osmotic stress conditions at each level of PEG 6000, and very few breeding lines were able to outperform the referent genotypes in the PEG 600 treatments. However, SI results for root number obtained from several breeding lines were higher than those of the referent genotypes in the D-mannitol treatments.

Changes in rooting parameters under stress conditions can vary; for example, each observed rooting trait (root number and root length) decreased as D-mannitol concentration increased [32], but the root number of cv. Boró (a highly drought-tolerant variety) increased at the 0.2 M D-mannitol level. Similarly, the root dry mass increased by 25% on average in 43 potato genotypes [34]. Moreover, when Zaki and Radwan [33] tested the osmotic stress tolerance of 21 potato cultivars on media containing sorbitol at three concentration levels (0.1, 0.2 and 0.3 M), they also found that tolerant genotypes developed greater or slightly

decreased root mass under stress compared to the sensitive cultivars, which suffered from strong inhibition in their root development. They also observed that stimulated root growth occurred frequently at the lowest level of stress, and sometimes it could be detected at the mid-level of stress.

Opposite results observed and published in terms of root parameters such as root length, root dry mass, and root number [19,20] may be attributed to the fact that some—tolerant—varieties can respond to drought stress with increased root length, while root length does not change or decrease in more sensitive varieties compared to referent lines [36]. In addition, different experimental settings and interactions between genotypes and environment can also contribute to the variability of results [19].

In general, drought had a greater effect on the above-ground than under-ground growth in potato crop [36,55], and in the case of other crops [59–61]. In vitro experiments with potatoes yielded similar results in osmotic stress-induced changes in shoots and root systems [32,34]. In our experiments, shoot growth was also more inhibited than the growth of the root system. SI values for all treatments and all breeding lines averaged 40.06, 60.79, 76.02 and 82.61 for SL, RL, SR and RN, respectively (Table 1).

Despite a significant reduction detected in shoot length, this alone did not appear to be an appropriate trait for grouping genotypes by their osmotic stress tolerance, because high SI values of shoot lengths were not regularly accompanied by higher survival rates in breeding lines. A similar conclusion was reached by other researchers who did not recommend the use of changes in shoot length as a suitable parameter for selection, for example, in wheat [62] or in potato [32].

However, potato clones did not show the same reactions to different osmotic agents. Responses observed on media supplemented with PEG 6000 and D-mannitol were sometimes similar, but most frequently the performance of potato shoot cultures varied on different media considering the type of osmotic material as well as its concentration. In addition, the evaluation of potato genotypes on media with PEG 600 seemed to be difficult because of the very high levels of inhibition detected in breeding lines. Gangopadhyay et al. [63] also found that considering the growth, viability and proline content of tobacco (*Nicotiana tabacum* L. *var*. Jayasri), the responses of callus lines depended on physico-chemical characters of the used osmotic agents (PEG 6000, D-mannitol and NaCl).

The various responses of genotypes may be due to different effects of the three osmotic agents (PEG 6000, D-mannitol and PEG 600) on the growth and developmental characters of in vitro shoot cultures. In fact, we used PEG 6000, which is a non-ionic, non-penetrating osmotic substance due to its high molecular weight [63]. In contrast, D-mannitol is a sugar alcohol that is also a non-ionic but penetrating osmotic agent [31]. PEG 600, being less than 1000 in molecular weight, is a non-ionic osmotic, but due to its lower molecular weight, it is more likely to be absorbed by plants and may have toxic effects [64].

As we observed, they all inhibited shoot growth, and the already quite strong inhibition further increased with increasing concentrations of these osmotic agents. In contrast, the root length was inhibited and also stimulated when explants were grown on media supplemented with the lowest levels of PEG 6000 or D-mannitol, whereas an inhibitory effect was observed in the case of PEG 600. Moreover, the number of roots were often significantly increased by PEG 6000 and D-mannitol treatments, but this was not true for treatments with PEG 600. Survival rates were also decreased by each treatment.

Even though PEG can result in serious stress to plants, it is used frequently as an osmotically active agent [51]. In our experiments the significantly strongest inhibition on studied characters was observed on shoot cultures grown on media with PEG 600. PEG 6000 and D-mannitol had a broadly similar inhibitory effect on plantlets, except for the number of roots, where the presence of D-mannitol resulted in a significantly lower SI value compared to PEG 6000 in the mean of all concentrations and breeding lines.

According to results reported by Thimann et al. [65], a very small amount of exogenous D-mannitol was able to enter potato disc tissues. In contrast, Trip et al. [31] found that potato leaf discs absorbed D-mannitol in a very large proportion (99%), although only 1.3%

was metabolized. In potato tissue cultures, Lipavská and Vreugdenhil [66] revealed that in vitro potato shoots can readily absorb D-mannitol from the medium, and its transport to shoots was unobstructed as well. In spite of these results, in experiments involving potatoes, D-mannitol was also used to induce osmotic stress. The level that could be applied to distinguish genotypes was higher (0.8 M) for calli culture than that for shoot culture (0.2–0.4 M) [67,68]. The researchers detected strong inhibition in growth and survival with 0.4 M D-mannitol, even in the case of tolerant genotypes. Thus, we tested the effect of D-mannitol at concentrations of 0.1, 0.2 and 0.3 M, the last of which also led to strong inhibition of shoot length but had a weaker inhibitory effect on survival. Evers et al. [39] found the same tendencies when 0.2 and 0.3 M D-mannitol were applied in experiments with *Solanum phureja* and *S. tuberosum* clones.

In addition to variations in the tolerance of breeding lines to osmotic stress, there might be some differences in their ability to absorb D-mannitol, and/or tolerate the toxic effect of PEG 600; thus, interactions between genotypes and osmotic agents added to medium could lead to various responses. Significant interactions between genotypes and the degree of osmotic pressure also should be considered [40].

5. Conclusions

All tested parameters were affected by each osmotic material used in our experiments. In general, shoot length was the most inhibited, while changes in rooting parameters both stimulated and reduced growth, depending on the genotype. Usually, the survival rates decreased significantly in treatments with strong osmotic pressure. Responses of genotypes were affected by the type and concentration of osmotic agent. Comparing genotypes, we can conclude that C103 tolerated more osmotic stress than C107, although both of them are drought-tolerant. Out of the 27 total breeding lines examined, nine genotypes (C8, C63, C14, C2, C5, C58, C12, C11 and C30) were shown to be worthy of further investigation.

Besides their high survival rate SI values, their rooting parameters were stimulated or hardly inhibited comparing to other genotypes, and their shoot length SI values were also high in several cases. We found significant interactions between genotypes and osmotic agents and their concentrations. In fact, breeding lines with high survival rates showed high SI values in the PEG 6000 or D-mannitol treatment or in both, but not in treatments with PEG 600. In general, the PEG 6000 10.0% and D-mannitol 0.2 M treatments proved to be the most suitable for differentiating genotypes according to their osmotic stress tolerance. The PEG 6000 and D-mannitol-containing media were tested at different concentrations, but they resulted in very similar inhibitory effects in terms of shoot length, root length, and survival rate (Table 1). In fact, the rate of absorbed and metabolized D-mannitol is not known. The very strong inhibitions observed in the PEG 600 treatments may be attributable mainly to its toxicity, because their resulting osmolality values were higher than those of PEG 6000 treatments but lower than those of media with D-mannitol (Table S5). However, a significant root growth stimulating effect was also observed in the referent lines, which is probably due to the adaptation response to osmotic stress. There are ongoing in vivo greenhouse and field experiments with selected breeding lines to confirm their drought tolerance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8070591/s1, Figure S1: Survival rate SI (SI_{SR}) values of potato genotypes under osmotic stress induced by PEG 6000 added to the medium at levels of 5.0% (c), 7.5% (b) and 10.0% (c). Figure S2: Shoot length SI values (SI_{SL}) of potato genotypes under osmotic stress induced by PEG 6000 added to the medium at levels of 5.0% (a), 7.5% (b) and 10.0% (c); Figure S3: Root length SI (SI_{RL}) values of potato genotypes under osmotic stress induced by PEG 6000 added to the medium at levels of 5.0% (a), 7.5% (b) and 10% (c); Figure S4: Root number SI (SI_{RN}) values of potato genotypes under osmotic stress induced by PEG 6000 added to the medium at levels of 5.0% (a), 7.5% (b) and 10% (c).; Figure S5: Survival rate SI (SI_{SR}) values of potato genotypes under osmotic stress induced by PEG 600 added to the medium at levels of 2.5% (a), 5.0% (b) and 7.5% (c); Figure S6: Shoot length SI (SI_{SL}) values of potato genotypes under stress treatment induced

by PEG 600 added to the medium at levels of 2.5% (a), 5.0% (b) and 7.5% (c).; Figure S7: Root lengths SI (SI_{RL}) values of potato genotypes under osmotic stress induced by PEG 600 added to the medium at levels of 2.5% (a), 5.0% (b), 7.5% (c); Figure S8: Root number SI (SI_{RN}) values of potato genotypes under osmotic stress induced by PEG 600 added to the medium at levels of 2.5% (a), 5.0% (b), 7.5% (c); Figure S9: Survivor rate SI (SI_{SR}) values of potato genotypes under stress treatment induced by D-mannitol added to the medium at levels of 0.1 M (a), 0.2 M (b) and 0.3 M (c); Figure S10: Shoot length SI (SI_{SL}) values of potato genotypes under stress treatment induced by D-mannitol added to the medium at levels of 0.1 M (a), 0.2 M (b) and 0.3 M (c); Figure S11: Root length SI (SI_{RL}) values of potato genotypes under stress treatment induced by D-mannitol added to the medium at levels of 0.1 M (a), 0.2 M (b) and 0.3 M (c); Figure S12: Root number SI (SI_{RN}) values of potato genotypes under stress treatment induced by D-mannitol added to the medium at levels of 0.1 M (a), 0.2 M (b) and 0.3 M (c).; Table S1: Ranking of breeding lines cultured on medium supplemented with PEG 6000 including each SI value; Table S2: Ranking of breeding lines cultured on medium supplemented with PEG 600 including each SI value; Table S3: Ranking of breeding lines cultured on medium supplemented with D-mannitol including each SI value; Table S4: Ranking of breeding lines cultured on medium supplemented with PEG 6000, PEG 600 or D-mannitol including each SI value; Table S5: Osmolality values of MS media supplemented with different osmotic agents used in experiments.

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